brief communication

On the analysis of high order moments of fluorescence fluctuations

Hong Qian and Elliot L. Elson
Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110

ABSTRACT A simple, straightforward analysis to characterize the distribution of aggregate sizes in a reversible aggregation system at equilibrium is presented. The method, an extension of fluorescence correlation spectroscopy (FCS), is based on measurements of higher order moments of spontaneous fluctuations of fluorescence intensity emitted from a defined open region of the sample. These fluctuations indicate fluctuations of the numbers of the fluorescent molecules in the observation region. Shot noise resulting from the random character of fluorescence emission and from the photoelectric detection system is modeled as a Poisson distribution and is subtracted from the measured photon count fluctuation moments to yield the desired fluorescence fluctuation moments. This analysis can also be used to estimate the fraction of immobile fluorophores in FCS measurements.

INTRODUCTION

Determining the size distribution of molecular aggregates and polymers has been a problem of interest to polymer chemists and biophysists for decades (Flory, 1953). Especially in reversible polymerization and aggregation systems such as the formation of micelles or cytoskeletal, e.g., actin, filaments, it is essential to use a nonperturbing method to determine the molecular weight distribution (Frieden, 1985). In a recent series of papers, Palmer and Thompson (1987, 1989*a*, *b*) have introduced high order fluorescence correlation analysis to measure aggregation of fluorescent molecules. This method, an extension of conventional fluorescence correlation spectroscopy (FCS; Elson, 1985; Elson and Webb, 1975), is based on an analysis of the fluctuations in fluorescence that result when the molecular aggregates diffuse into or out of a small open region of the system. Whereas conventional FCS measures transport and chemical reaction rates and also yields limited information about aggregate sizes from a simple autocorrelation of fluorescence fluctuations (cf. Magde et al., 1974; Petersen, 1986), Palmer and Thompson have demonstrated that further information about the distribution of aggregate sizes may be obtained from higher moments of the fluorescence fluctuations. The analysis of the fluorescence fluctuations is complicated theoretically and experimentally by the contribution of shot noise to the measured fluctuation moments. In this note we present an alternative analysis of this problem, which is also overall a simplification. We first discuss the moments of the fluorescence intensity distribution and their relationship to the distribution of aggregate sizes. Then we present a straightforward, convenient, and practical way of accounting for shot noise.

BASIC THEORY OF THE ANALYSIS OF HIGHER FLUCTUATION MOMENTS

The determination of the distribution of degrees of aggregation depends only on the equilibrium properties of the system and uses only statistical information about the measured fluorescence intensity fluctuations. The time correlation of the fluctuations plays no essential role in the analysis but provides a means for excluding shot noise. The basic concept for this approach originated with Smoluchowski in 1910 (Brenner et al., 1978).

It is straightforward to write down the fluctuation moments for the fluorescence intensity supposing that the particles in the observation region are governed by a Poisson distribution. In dealing with nonuniform observation regions (i.e., nonuniform excitation profiles), however, there are advantages to taking a more general approach, as follows. We denote the random variable Φ as the integrated fluorescence intensity from a well-defined volume of the sample and time interval. For a single fluorescent molecule at position r with fluorescence yield q, $\Phi = qI(r)$, where $I(r)$ is the distribution of fluorescence intensity, which depends on the excitation intensity and the fluorescence photon collection characteristics of the optical measurement system (Koppel et al., 1976). Therefore (Feller, 1957)

$$\langle \Phi^n \rangle_1 = q^n \int_V I^n(r) P(r)\, \mathrm{d}r,$$

where V is the total sample volume, and $\langle \cdot \rangle_1$ indicates an ensemble average for the system containing only one particle. Since the molecule diffuses freely throughout the system, the equilibrium probability is uniformly distrib-

0006-3495/90/02/375/06 $2.00

uted, i.e., $P(r) = 1/V$,

$$\langle \Phi^n \rangle = (q^n/V) \int_V I^n(r)\, dr,$$

therefore,

$$\begin{aligned}
\langle \Phi \rangle_1 &= \chi_1 q/V, \\
\langle (\Delta\Phi)^2 \rangle_1 &= \langle \Phi^2 \rangle - \langle \Phi \rangle^2 \\
&= [\chi_2/V - (\chi_1/V)^2] q^2, \\
\langle (\Delta\Phi)^3 \rangle_1 &= [\chi_3/V - 3\chi_2\chi_1/V^2 \\
&\quad + 2(\chi_1/V)^3] q^3, \\
\langle (\Delta\Phi)^4 \rangle_1 - 3\langle (\Delta\Phi)^2 \rangle_1^2 &= (\chi_4/V - 4\chi_1\chi_3/V^2 - 3\chi_2^2/V^2 \\
&\quad + 12\chi_2\chi_1^2/V^3 - 6\chi_1^4/V^4) q^4,
\end{aligned}$$

where

$$\chi_n = \int_V I^n(r)\, dr$$

which are numerical parameters that describe the excitation and collection characteristics of the optical measurement system (Palmer and Thompson, 1989*b*). It can be shown that the above expressions are the first four cumulants of the random variable Φ (Aitken, 1957). If rather than one there are now N identical and independent, noninteracting particles in the system, the cumulant for the random variable Φ, the total fluorescence intensity from N particles, will simply be the sum of the cumulants associated with the individual particles (Aitken, 1957). That is

$$\begin{aligned}
\langle (\Delta\Phi)^2 \rangle_N &= N\langle (\Delta\Phi)^2 \rangle_1, \\
\langle (\Delta\Phi)^3 \rangle_N &= N\langle (\Delta\Phi)^3 \rangle_1, \\
\langle (\Delta\Phi)^4 \rangle_N - 3\langle (\Delta\Phi)^2 \rangle_N^2 &= N[\langle (\Delta\Phi)^4 \rangle_1 - 3\langle (\Delta\Phi)^2 \rangle_1^2],
\end{aligned}$$

where $\langle \cdot \rangle_N$ represents the ensemble average for a system containing N particles. In most experimental conditions, the excitation illuminated volume $[\sim \int I^n(r)dr/I^n(0)]$ is much smaller than the total sample volume V, the limiting case ($1/V \rightarrow 0$) required for the validity of the Poisson distribution. Hence terms of higher order in $1/V$ can be neglected. When there are a total of N molecules in V, $\bar{c} = (N/V)$ is the number concentration of molecules.

Now suppose that in a multicomponent system the mean concentration of the kth component is $\bar{c}_k$ with fluorescent yield q_k. Suppose also that the solution is ideal, i.e., molecular interactions are negligible. Then the overall fluctuation moments for the fluorescence intensity will be

$$\begin{aligned}
\langle \Phi \rangle &= \chi_1 \Sigma q_k \bar{c}_k, \\
\langle (\Delta\Phi)^2 \rangle &= \chi_2 \Sigma q_k^2 \bar{c}_k, \\
\langle (\Delta\Phi)^3 \rangle &= \chi_3 \Sigma q_k^3 \bar{c}_k, \\
\langle (\Delta\Phi)^4 \rangle - 3\langle (\Delta\Phi)^2 \rangle^2 &= \chi_4 \Sigma q_k^4 \bar{c}_k.
\end{aligned}$$

These results may also be derived directly by assuming a Poisson distribution of the fluorescent particles in the illuminated volume. Clearly, for a two-component system, $\bar{c}_1$, q_1, and $\bar{c}_2$, q_2, we can determine the equilibrium ratio of the components by measuring $\langle \Phi \rangle$, $\langle (\Delta\Phi)^2 \rangle$, and $\langle (\Delta\Phi)^3 \rangle$.

FCS experiments are typically carried out using a focused laser beam with Gaussian intensity profile to excite fluorescence and define the observation region. The divergence of the beam above and below the plane of focus leads to complexity of interpretation that has been analyzed elsewhere (Qian and Elson, 1989*b*). For simplicity we suppose that the fluorescent molecules are confined to the plane of focus of the laser excitation beam. Hence it is sufficient to take $I(r) = I_0 \exp[-2r^2/\omega_0^2]$ with $\exp(-2)$ radius ω_0. Thus, $\chi_n = I_0^n(\pi\omega_0^2)/2n$ and for a system containing only one component

$$\langle \Phi \rangle = q\bar{c}\chi_1 = q\bar{c}(I_0/2)(\pi\omega_0^2) = q\langle N \rangle I_0/2,$$

where $\langle N \rangle$ is the mean number of particles in the $(\pi\omega_0^2)$ area. Similarly,

$$\begin{aligned}
\langle (\Delta\Phi)^2 \rangle &= q^2\bar{c}(I_0/2)^2(\pi\omega_0^2) = \langle N \rangle (qI_0)^2/4, \\
\langle (\Delta\Phi)^3 \rangle &= \langle N \rangle (qI_0)^3/6
\end{aligned}$$

so we have

$$\langle (\Delta\Phi)^2 \rangle / \langle \Phi \rangle^2 = 1/\langle N \rangle$$

as previously derived (Elson and Magde, 1974).

Suppose we have a mixture of molecular aggregates in which there are N_k aggregates containing k monomers. We can characterize the distribution of aggregate sizes, $\{N_k\}$, in terms of the moments of the distribution. For example, the mean, μ, and standard deviation, σ^2, of the distribution are

$$\mu = \Sigma k N_k / \Sigma N_k, \quad \sigma^2 = \Sigma k^2 N_k / \Sigma N_k - \mu^2.$$

We can relate this to FCS measurements carried out with a Gaussian laser excitation beam, as follows:

$$\frac{\langle (\Delta\Phi)^2 \rangle}{\langle \Phi \rangle^2} = \frac{\Sigma k^2 N_k}{N_t \cdot \Sigma k N_k} = \frac{\sigma^2 + \mu^2}{\mu \cdot N_t}.$$

N_t is the mean number of total monomers in beam. This result was previously derived by Petersen (1986). More detailed characterization of the distribution is obtained by including higher moments in the analysis (Palmer and Thompson, 1987), and derivations are straightforward.

THE CONTRIBUTION OF SHOT NOISE

We have derived the relation between the distribution of aggregate size and high moments of fluorescence intensity Φ. Experimentally, however, the directly measurable quantity is the fluorescence photon counts. The moments calculated from fluorescence photon counts are not identical to those of the fluorescence intensity because of the randomness in the emission of fluorescence photons after excitation and the characteristics of photon detection by a photomultiplier. This effect, commonly called shot noise, adds an overwhelming contribution to the zero time amplitude of the fluorescence autocorrelation functions measured in FCS experiments and, therefore, to the moments needed to characterize aggregation distributions. Hence, it is necessary to exclude shot noise from the experimental measurements.

Under constant excitation intensity, both the fluorescence photon emission and the generation of the photoelectron cascade in the photomultiplier are random, usually following a Poisson distribution (Mandel et al., 1964; Saleh, 1978). This instantaneous randomness contributes to the measured shot noise. Both the power spectrum of the instantaneous fluorescence ($\Delta\omega \simeq 10^{14}$ s^{-1}) and the frequency response of the photomultiplier ($\Delta\omega \simeq 10^{-8}\,s^{-1}$) are sufficiently broad that the correlation of the shot noise persists only over nanoseconds in contrast to our characteristic measurement time ($>\sim 10^{-4}$ s). Thus, the shot noise contributes only to the zero time correlation, and decays to zero before the first time point in the measured correlation function.

A general way to determine $\langle(\Delta\Phi)^2\rangle$ is by measuring the photon count autocorrelation function, $G_1(t)$, in a conventional FCS experiment and then extrapolating to zero time to obtain the initial amplitude of the fluorescence autocorrelation function $F_1(0) = \langle(\Delta\Phi)^2\rangle$ (Icenogle and Elson, 1985). This method is, however, time consuming and requires a large amount of calculation and curve fitting, and also a priori knowledge of the functional form of $G_1(t)$. Furthermore, data of increasingly high quality is required to evaluate satisfactorily the higher order correlation functions. We have developed an alternative approach to this problem by directly calculating the moments of the photon counts and explicitly correcting the instantaneous shot noise effect.

The properties of the higher order time correlations are similar to those of the conventional FCS fluorescence autocorrelation function but more complex. In principle, the higher order correlation functions have more than one time argument. When these time arguments are all different from each other, there is no contribution from shot noise to the measured correlation function. Therefore, the photon count correlation $G(\cdot)$ and fluorescence intensity correlation $F(\cdot)$ are identical (within a constant prefactor)

$$G_1(t_1) = q^2F(t_1),$$
$$G_2(t_1, t_2) = q^3F(t_1, t_2),$$
$$G_3(t_1, t_2, t_3) = q^4F(t_1, t_2, t_3),$$

where $0 < t_1 < t_2 < t_3$, $t_1 \neq t_2$, $t_2 \neq t_3$. But when $t_1 = t_2$, the shot noise does play a role and, therefore, (Qian and Elson, manuscript in preparation)

$$\lim_{t_1 \to 0} G_1(t_1) \neq G_1(0)$$

and likewise,

$$\lim_{t_2 \to t} G_2(t, t_2) \neq G_2(t, t),$$
$$\lim_{t \to 0} G_2(t, t) \neq G_2(0, 0).$$

Therefore, when the high order time correlations are defined as $G_2(t, t)$, $G_3(t, t, t)$, etc., and the moments are obtained by extrapolation: $\lim_{t \to 0} G_n(t, t, \ldots)$, the analysis is substantially complicated by the need to correct for the shot noise contribution. The high moments of the fluorescence intensity distribution can be obtained without shot noise by the following extrapolation:

$$\lim_{t_1 \to 0} \lim_{t_2 \to 0} G_2(t_1, t_2), \quad t_1 \neq t_2.$$

But this method, as stated before, requires extensive computation to evaluate the two-dimensional $G(t_1, t_2)$, and also requires a priori knowledge of the functional form for $G(t_1, t_2)$.

Alternatively, we have developed a simple and practical approach to directly estimate the shot noise contribution by assuming the following Poisson distribution of photon counts P for a single fluorescent particle

$$\text{Prob}\{P = k\} = \int_V \frac{(\lambda I(r)T)^k}{k!} \exp\left(-\lambda I(r)T\right) \frac{dr}{V},$$

where λ is the rate constant for photoelectron generation, which is proportional to quantum yield of fluorophore and photon detection device; $I(r)$ is the intensity of excitation light; and T is the data collecting dwell time. An emission-detection statistical distribution can also be experimentally evaluated.

By using this random response model, we have obtained the moments of the distribution of photon counts P (Saleh, 1978; Qian and Elson, manuscript in preparation):

$$\langle P\rangle = \langle\Phi\rangle, \tag{1a}$$
$$\langle(\Delta P)^2\rangle = \langle P\rangle + \langle(\Delta\Phi)^2\rangle, \tag{1b}$$

and

$$\langle(\Delta P)^3\rangle = 3\langle(\Delta P)^2\rangle - 2\langle P\rangle + \langle(\Delta\Phi)^3\rangle. \quad (1c)$$

The terms $\langle P\rangle$ in Eq. 1 *b* and $3\langle(\Delta P)^2\rangle - 2\langle P\rangle$ in (1*c*) are the shot noise contributions. Hence the moments of the fluorescence fluctuations may be readily obtained by sequentially subtracting the shot noise terms. The intensity dependence of the shot noise effect is clear. Since $\langle\Phi\rangle \propto Q\ (=\lambda I_0 T)$, a constant proportional to the total excitation power, the fluorophore quantum yield, and the dwell time of the measurement, and $\langle(\Delta\Phi)^n\rangle \propto Q^n$, when Q is large, the first term in Eq. 1 *b* and first two terms in Eq. 1 *c* will be negligible (Palmer and Thompson, 1989*a*). We have experimentally compared the evaluation of shot noise obtained as above and by extrapolation of the correlation functions to zero time, and have verified their agreement (Qian, data not shown).

As pointed out by Palmer and Thompson (1989*a*), there can also be random contributions from other sources in the process of photon collection and detection. Then theoretical prediction is difficult, and an empirical response function must be introduced. This subject has been extensively studied (Sarantites et al., 1980; Palmer and Thompson, 1989*a*).

CALCULATION OF AN IMMOBILE FRACTION FROM MOMENTS

In systems containing particles with a wide range of mobilities, the slower moving particles may appear to be immobile under conditions in which the motion of the more rapidly moving particles is observable. This immobile fraction is readily measured in fluorescence photobleaching recovery (FPR) measurements (e.g., Petersen et al. 1986), and has been observed in many different kinds of biological systems (e.g., Schlessinger et al., 1976). The conventional FCS experiment does not yield the fraction of immobile particles. Nevertheless, there are situations, e.g., at very low fluorophore concentrations or when fluctuations are very large due to the grouping of a large number of fluorophores together in a small number of particles, when FCS may prove more suitable than FPR. Advantages and disadvantages of both methods have been studied (Icenogle and Elson, 1985). Hence it is desirable to have a method to obtain the immobile fraction from FCS measurements. This can be accomplished by introducing the third moment of fluorescence fluctuations and treating the system as having two components, one mobile and one immobile. (This argument is for a self-contained experiment. If an external calibration of q already exists, then only the first two moments are required to determine the immobile fraction: $\langle N_m\rangle / \langle N_t\rangle = q^{-1}(\chi_1/\chi_2)\langle(\Delta\Phi)^2\rangle/\langle\Phi\rangle$.)

Consider a small open region ($\pi\omega_0^2$) in a mobile–immobile system. The number of mobile particles in this region follows Poisson distribution: $\langle N_m\rangle = \langle(\Delta N_m)^2\rangle = \langle(\Delta N_m)^3\rangle$ = average number of mobile particles in the observation region. For the immobile particles, however

$\langle N_n\rangle$ = number of immobile particles in the observation region

$$\langle\Delta N_n\Delta N_n\rangle = \langle\Delta N_n\Delta N_n\Delta N_n\rangle = 0.$$

If these particles are uniformly labeled with fluorophore, and the mobile component N_m and the immobile component N_n are independent, the moments of fluorescence intensity are:

$$\begin{aligned}\langle\Phi\rangle &= \chi_1 q(\langle N_m\rangle + \langle N_n\rangle),\\ \langle(\Delta\Phi)^2\rangle &= \chi_2 q^2[\langle(\Delta N_m)^2\rangle + \langle(\Delta N_n)^2\rangle]\\ &= \chi_2 q^2\langle(\Delta N_m)^2\rangle\\ &= \chi_2 q^2\langle N_m\rangle.\end{aligned}$$

Hence the immobile component does not contribute to the fluctuation, and similarly,

$$\langle(\Delta\Phi)^3\rangle = \chi_3 q^3\langle N_m\rangle. \quad (2)$$

For a single component system, the reciprocal of the amplitude of the normalized autocorrelation function yields the total number of particles in the observation region (Elson and Magde, 1974). When immobile particles are present, however,

$$\begin{aligned}\langle\Phi\rangle^2/\langle(\Delta\Phi)^2\rangle &= (\chi_1^2/\chi_2)(\langle N_m\rangle + \langle N_n\rangle)^2/\langle N_m\rangle\\ &= (\chi_1^2/\chi_2)\langle N_t\rangle^2/\langle N_m\rangle, \quad (3)\end{aligned}$$

where $\langle N_t\rangle = \langle N_m\rangle + \langle N_n\rangle$ is the total number of particles, mobile and immobile, in the observation region. When $\langle N_n\rangle = 0$ this ratio yields $\langle N_t\rangle$, and when $\langle N_m\rangle = 0$ it becomes infinite. By the same analysis we have

$$\langle\Phi\rangle^3/\langle(\Delta\Phi)^3\rangle = (\chi_1^3/\chi_3)\langle N_t\rangle^3/\langle N_m\rangle.$$

Combining Eqs. 2 and 3, we have

$$\langle N_m\rangle/\langle N_t\rangle = (\chi_3\chi_1/\chi_2^2)\langle(\Delta\Phi)^2\rangle^2/[\langle\Phi\rangle\langle(\Delta\Phi)^3\rangle].$$

For a two-dimensional Gaussian laser excitation intensity profile, $(\chi_3\chi_1/\chi_2^2) = 4/3$. Computer simulation has confirmed this conclusion (Qian, unpublished results). More important, for a three-dimensional system, $(\chi_3\chi_1/\chi_2^2)$ is measured to be ~2 for our laser microscope system (Qian, data not shown). This agrees approximately with Palmer and Thompson (1989*b*), who obtain $(\chi_3\chi_1/\chi_2^2) \simeq 3$ for their system.

COMPARISON WITH FLUORESCENCE DISTRIBUTION SPECTROSCOPY (FDS)

An alternative approach to the same problem is to characterize the aggregation distribution $\{N_k\}$ directly from the measured distribution of fluorescence photon counts, $P_P(n)$, rather than from its moments. There are at least three ways to approach this task. First, one can calculate $\{N_k\}$ directly by inverting the response transformation (Qian and Elson, 1989*a*). This scheme is theoretically attractive, but computationally difficult. One may also presuppose a specific form for $\{N_k\}$, and calculate the parameters that give the best fit to experimentally measured $P_P(n)$. This is somewhat similar to moment analysis. In some extreme cases, the distribution $P_P(n)$ can directly show multiple peaks, providing qualitative information about aggregates (Qian and Elson, 1989*a*). Moment analysis and distribution analysis are complementary. While moment analysis is highly sensitive to aggregation, it is also strongly affected by external noise; distribution analysis is less sensitive to both aggregation state and noise.

DISCUSSION

The moments of fluorescence fluctuation depend only on the equilibrium properties of the measured system. Nevertheless, the dynamic properties of the system are important in designing an experimental measurement. If diffusion of the fluorescent aggregates is sufficiently fast, a sufficiently large sample of fluctuations may be obtained by observing spontaneous diffusion fluctuations in solution (Palmer and Thompson, 1987). For slowly moving or immobile particles, spatial fluctuations in concentration can be measured by systematic scanning (Weissman et al., 1976; Petersen, 1986) or flow (Magde et al., 1978). A straightforward moment analysis theory is presented here, which is simpler than the previous treatment (Palmer and Thompson, 1987), and should be easier to apply. Complications caused by shot noise contributions are avoided without resorting to lengthy time correlation calculations by invoking a response function to model the random characteristics of photon emission and collection. High moment analysis is especially sensitive to the excitation intensity profile. Therefore careful characterization of the laser fluorescence microscope optical systems used in these experiments is essential (Qian and Elson, 1988; Palmer and Thompson, 1989*b*; Qian and Elson, 1989*b*). High moment analysis can also provide an estimation of the immobile fraction in a sample, a parameter which has been commonly used in FPR. High moment analysis, especially when combined with FDS, can provide unique information about molecular aggregation.

We would like to thank Drs. George Drysdale, Carl Frieden, Howard Reiss, and Robert Yaris for their encouragements in the process of this work. We also thank Mike Kolodney for reading the manuscript and for helpful discussions.

This work was supported by National Institutes of Health grant GM38838.

Received for publication 31 July 1989.

REFERENCES

Aitken, A. C. 1957. Statistical Mathematics. Oliver and Boyd, Ltd. London. 153 pp.

Brenner, S. L., R. J. Nossal, and G. H. Weiss. 1978. Number fluctuation analysis of random locomotion: statistics of a Smoluchowski process. *J. Stat. Phys.* 18:1–18.

Elson, E. L. 1985. Fluorescence correlation spectroscopy and photobleaching recovery. *Annu. Rev. Phys. Chem.* 36:379–406.

Elson, E. L., and D. Magde. 1974. Fluorescence correlation spectroscopy. I. Conceptual basis and theory. *Biopolymers.* 13:1–27.

Elson, E. L., and W. W. Webb. 1975. Concentration correlation spectroscopy: a biophysical probe based on occupation number fluctuations. *Annu. Rev. Biophys. Bioeng.* 4:311–334.

Feller, W. 1957. An introduction to probability theory and its applications. John Wiley and Sons, New York. 461 pp.

Flory, P. 1953. Principles of Polymer Chemistry. Cornell Press, New York. 672 pp.

Frieden, C. 1985. Actin and tubulin polymerization: the use of kinetic methods to determine mechanism. *Annu. Rev. Biophys. Biophys. Chem.* 14:189–210.

Icenogle, R. D., and E. L. Elson. 1985. Fluorescence correlation spectroscopy and photobleaching recovery of multiple binding reactions. I. Theory and FCS measurements. *Biopolymers.* 22:1919–1948.

Koppel, D. E., D. Axelrod, J. Schlessinger, E. L. Elson, and W. W. Webb. 1976. Dynamics of fluorescence marker concentration as a probe of mobility. *Biophys. J.* 16:1315–1329.

Magde, D., E. L. Elson, and W. W. Webb. 1974. Fluorescence correlation spectroscopy. II. An experimental realization. *Biopolymers.* 13:29–61.

Magde, D., W. W. Webb, and E. L. Elson. 1978. Fluorescence correlation spectroscopy. III. Uniform translation and laminar flow. *Biopolymers.* 17:361–376.

Mandel, L., E. C. G. Sudarshan, and E. Wolf. 1964. Theory of photoelectric detection of light fluctuations. *Proc. Phys. Soc. (Lond.).* 84:435–444.

Palmer, A. G., and N. L. Thompson. 1987. Molecular aggregation characterized by high order autocorrelation in fluorescence correlation spectroscopy. *Biophys. J.* 52:257–270.

Palmer, A. G., and N. L. Thompson. 1989*a*. Intensity dependence of high-order autocorrelation function in fluorescence correlation spectroscopy. *Rev. Sci. Instrum.* 60:624–633.

Palmer, A. G., and N. L. Thompson. 1989*b*. Optical spatial intensity profiles for high order autocorrelation in fluorescence spectroscopy. *Appl. Opt.* 28:1214–1220.

Petersen, N. O. 1986. Scanning fluorescence correlation spectroscopy. I.

Theory and simulation of aggregation measurements. *Biophys. J.* 49:809–815.

Petersen, N. O., S. Felder, and E. L. Elson. 1986. Measurement of lateral diffusion by fluorescence photobleaching recovery. *Handb. Exp. Immunol.* 1:24.1–24.23.

Qian, H., and E. L. Elson. 1988. 3-D fluorescence correlation spectroscopy in bulk solution. *Proc. Soc. Photo-opt. Instr. Eng.* 909:352–359.

Qian, H., and E. L. Elson. 1989*a*. Characterization of the equilibrium distribution of polymer molecular weights by fluorescence distribution spectroscopy (theoretical results). *Appl. Polym. Symp.* 43:305–314.

Qian, H., and E. L. Elson. 1989*b*. Characterization of confocal laser based microscope by digital image analysis: an optical sectioning microscopy approach. *In* Digitized Video Microscopy. B. Herman and K. Jacobson, editors. Alan R. Liss, Inc., New York. In press.

Saleh, B. 1978. Photoelectron Statistics. Springer-Verlag, Berlin. 441 pp.

Sarantites, D. G., R. Lovett, and R. Woodward. 1980. A "nuclear-spin spectrometer": theory and general characteristics of a 4π gamma-ray multidetector for investigations of nuclear structure and reactions at high angular momentum. *Nucl. Instrum. Methods.* 171:503–519.

Schlessinger, J., D. E. Koppel, D. Axelford, K. Jacobson, W. W. Webb, and E. L. Elson. 1976. Lateral transport on cell membranes: mobility of con A receptors on myoblasts. *Proc. Natl. Acad. Sci. USA.* 73:2409–2413.

Weissman, M., H. Schindler, and G. Feher. 1976. Determination of molecular weights by fluctuation spectroscopy: application to DNA. *Proc. Natl. Acad. Sci. USA.* 73:2776–2780.